

## **REVUEW**

of the dissertation titled:

**"Properties and characteristics of newly isolated strains of lactic acid bacteria and application in model probiotic products for oral health "**,

for the acquisition of an educational and scientific degree "Doctor" in the field of higher education **5. Technical sciences, Professional direction - 5.11. Biotechnologies (DP.**

**Technology of biologically active substances**

of full-time doctoral student **Nikola Nikolov Atanasov,**

with supervisor: **Assoc. Prof. Dr. Dilyana Nikolova,**

By: **Prof. Dr. Svetla Danova, DS.c.,** Institute of Microbiology "Stefan Angelov" (IMicB),  
Bulgarian Academy of Sciences

### **ABOUT THE PROCEDURE:**

The current procedure for the acquisition of educational and scientific degree "Doctor" is conducted on the basis of Art. 4 of the law - ZRASRB, in connection with §7 of the Transitional and final provisions of the Regulations on the terms and conditions for acquiring scientific degrees and occupying academic positions at Sofia University "S<sup>t</sup>. Kl. Ohridski", According to the Order (*No. RD-38-264/31.05.2024*) of the Rector of Sofia University "St. Kliment Ohridski" I was elected as a member of the Scientific Jury and was designated as a reviewer at its first meeting. In my capacity as such, I declare that there is no conflict of interest within the meaning of §1, item 2a of the additional provisions of the RSARB between me and the candidate under the procedure for the educational and scientific degree "Doctor" and I am not subject to the restrictions under Art. 33 from the law- ZRASRB.

The absence of plagiarism in the candidate's scientific works has been proven according to the law.

### **RELEVANCE AND SIGNIFICANCE OF THE DEVELOPED PROBLEM:**

Human health has always been the focus of microbiological research, but due to the dynamics, increasing number and variability of abiotic and biotic factors, each new study contributes to the improvement of approaches to therapy and prevention. The doctoral student correctly assesses the role of the microbiome and focuses on researching beneficial characteristics of a group of microorganisms, which in recent years have been actively studied as a key factor in human health. The focus of my peer-reviewed scientific paper is on the role of probiotic lactic acid bacteria (LAB) in the oral microbiome. An innovative element of the development is the assessment of oral lactic acid microbiota, species diversity and biological activity as a health factor. Modulation of the oral microbiome is poorly studied, and the development provides new scientific information on an unsolved problem of the architecture and dynamics in distinct regions of the human microbiome and their relationship to health. All this gives me reason to evaluate the dissertation work as extremely relevant and significant. The PhD candidate successfully translates scientific fundamental to applied research, which is in

tune with the latest scientific research and market trends for healthy products, fulfilling the requirements of the scientific field 5.11 Biotechnology.

## **STRUCTURE AND VOLUME OF THE DISSERTATION**

The set of presented documents and materials, on paper and electronic variants, are very well prepared and meet all the legal requirements for obtaining scientific degrees and for holding academic positions at BF-SU. The dissertation is written according to the standard scheme in a volume of 149 pages. The requirements for form, content and ratio of the individual sections are met as follows: *Introduction* - 1 page, *Literature review* - 39 pages; *Purpose and tasks* - 2 pages; *Materials and methods* - 13 pages, *Results and discussion* - 48 pages, *Conclusions* - 2 pages, *Contributions* - 1 page. *References* - 26 pages; *Appendix* - 2 pages. The exact scientific style and grammatically correct Bulgarian language are impressive. Duly indicated abbreviations, together with the separate lists of tables and figures make it easier to read and evaluate the dissertation thesis. The work impresses with its logically consistent plan and clear concept for upgrading specific scientific results, as a basis for applied development. The dissertation is richly illustrated with 24 figures and 15 tables. A big number-466 literature sources in English and 1 in Bulgarian are cited, mainly from recent years, which shows excellent knowledge of the PhD student.

## **LITERARY AWARENESS AND STATEMENT OF GOAL AND OBJECTIVES**

The literature review is well structured, presents systematized scientific information and the latest achievements on the subject. It is closely linked logically to the subsequent “Results and Discussion” section. The human oral microbiome as a biotope and biocenosis with specific features are discussed in detail. Based on the biodiversity of microorganisms, a parallel is drawn with microbial balance and imbalance in the oral cavity and a connection between oral and systemic health. Information on probiotics and the main groups of lactic acid bacteria (LAB), recognized as such and their beneficial functional characteristics is presented in a systematic manner. The knowledge of the new LAB taxonomic classification and especially the changes in the order *Lactobacillus* makes an excellent impression. The good theoretical preparation and the knowledge of the latest findings on the problem are evident not only in the overview, but also in the overall presentation of the work. Nikola Atanasov uses this knowledge, including in the following section, in the selection of methodical approaches. An excellent impression is made by the analytical approach in the presentation of scientific information. The doctoral student summarizes it in figures, ex. such as fig. 5 - "*Potential Mechanisms of Action of Probiotics on Oral Health and Disease*". Thus, in an original way, he summarizes and constructs a complete rich literature reference, which provides the scientific basis for the specific experimental studies. The literature review directes logically to the objective and experimental tasks, which are clearly and precisely formulated. The aim of the thesis is well formulated: "*Isolation, identification and characterization of new strains of lactic acid bacteria from the oral microbiota, assessment of their probiotic potential, antimicrobial activities and investigation of their potential for application in a model probiotic product for oral health*". For its achievement, 7 concretely formulated tasks and 13 sub-tasks have been set.

## **EVALUATION OF MATERIALS AND METHODS USED**

The PhD student has selected and correctly presented a wide panel of classical research and modern molecular methods, in accordance with the ambitious work program and the multiple tasks. Knowledge and skills in selection and presentation are demonstrated. They are in very good logical sequence and relatedness. The fundamentals are the microbiological methods aimed at creating a control group of isolates from the microbiota of a sufficient group of volunteers (16 people without oral health problems); with subsequent selection of pure cultures of lactic acid bacteria (LAB). Nutrient media, cultivation conditions and morpho-physiological characteristics are skillfully selected and presented. I highly appreciate the polyphasic taxonomic panel combining classical microbiological methods and the gold standard in bacterial identification -16S rrn sequence analysis with the latest molecular methods of protein profiling by MALDI-TOF MS system and whole genome sequencing. The way of their presentation and especially the bioinformatics processing of the whole genomes prove the excellent knowledge of the theory and skillful inclusion for solving the tasks. They were selected for the experimental tasks of proving the genetic basis of the biological activity of the LAB.

The latest options for some of the *in vitro* protocols for characterizing candidate probiotic microorganisms were searched for. The selection of *in vitro* tests to assess the survival and development of the newly isolated LAB strains under simulated conditions of the different departments of the GIT is original. Special attention is given to factors in the oral cavity as follows: (i) survival in simulated oral conditions - incl. with oral stress; (ii) adhesive abilities of the tested LAB strains in simulated conditions; (iii) ability to bind to mucin; (iv) ability to form its own biofilm (v) antagonistic activity against test -pathogens, including oral (vi) *in vitro* evaluation of anti-biofilm activity against oral test pathogens, etc.

The PhD student chose a convenient and easily reproducible method for screening the antioxidant capacity, and the protocol was previously adapted for the study of LAB strains.

A very good impression is made by the accurate and detailed description of the methods and the protocols for their application, which enables other researchers to also apply them in their research. This also applies to the set of biotechnological approaches to evaluate the survival of the studied LAB strains during the lyophilization and storage process and the stability and survival in different formulations of a model oral health product. This is another proof of theoretical and experimental preparedness of the doctoral student. At each stage of experimental research, Nikola Atanasov looks for new, more discriminating and informative approaches and correctly applies statistical processing of information.

## **ASSESSMENT OF OBTAINED RESULTS AND CONTRIBUTIONS**

Overall, the dissertation is a voluminous, very serious work developed at a high scientific level. It evaluates important aspects of the species diversity and probiotic potential of lactic acid bacteria from the poorly studied oral microbiome. The doctoral student correctly focuses on the role of the entrance door for various microorganisms and, focusing on the lactic acid microbiota, brings out a complex molecular-biotechnological study. A sufficient number of 76 baseline samples from the oral cavity of 16 volunteers without the presence of pronounced oral diseases were collected. A total of 64 isolates were isolated from these samples and analyzed, using a classical processing approach. The combination of nutrient media and the scheme for isolation and species characterization are correct and very well logically constructed, successively passing from classical morpho-physiological and biochemical tests,

through genetic and sequencing analysis, comparing them with the protein identification profile of the MALDI ToF MS system. The original complex identification scheme was applied to 12 strains, and for 8 of them the latest taxonomic approach - whole genome sequencing - was used. I cannot fail to emphasize the enormous amount of work on the assembly of the genomes of these lactobacilli and the bioinformatic *in silico* analyses. The doctoral student shows knowledge and skills to perform not only the molecular techniques of DNA isolation, PCR detection of taxonomically and biologically important genes, but also bioinformatic analyses. They are not self-serving and conclusively substantiate the genetic bases of important probiotic and biotechnological properties in the selected group of active strains. Thus, the doctoral student achieves not only accurate species identification, but also reveals the cause-and-effect relationship between functionality and bacterial genome. I highly appreciate this approach, which can easily be refined as an original algorithm for functional evaluation of candidate-probiotic strains, in accordance with EFSA requirements. In this regard, the *in vitro* evaluation of the probiotic potential of the selected group of lactobacilli is rich in approaches and statistically reliable results. In them, the PhD student looks for a parallel with the ecological niche from which LABs were isolated and their functional role *in vivo*, without neglecting the established tests for resistance to GIT factors, mucin-binding, adhesive and biofilm-forming ability. Bioinformatic *in silico* analyzes of the whole genome sequences were carried out for the presence of genetic determinants for adhesin proteins responsible for the adhesive characteristics of the studied strains established in the *in vitro* analyses. Genes for two types of adhesion proteins have been demonstrated: adhesins, which help the adhesion of LAB to tissues and other cells, and lectins, which help LAB to bind to carbohydrates and other proteins. In the discussion to them, a link was competently found with the possible probiotic mechanism of these lactobacilli to prevent the invasion of pathogens *in vivo*. The experiments on the survival of the studied lactobacilli in simulated conditions of the oral cavity, survival under oral and gastrointestinal stress, auto- and co-aggregation ability and antagonistic activity are precisely performed and discussed. The author has specially selected a group of test-pathogens relevant to pathologies not only in the oral cavity. A complex approach was used to establish the antagonistic interactions of the studied strains, including antimicrobial activity of cell-free supernatants against a wide range of Gram-positive and Gram-negative test pathogens and against two oral test pathogens (*S. mutans* and *C. albicans*), as well as adhesive interactions with them. With the complementary *in silico* analysis of whole genome sequences, the presence of bacteriocin production mechanisms in some of the studied strains was demonstrated.

An excellent impression is made by the statistical processing (One-way ANOVA) of the results in the entire range of tests and the evaluation of the relationship between the established antagonistic properties in co-cultivation, co-aggregation and anti-biofilm activity obtained by Pearson's correlation analysis.

The overall experimental setup is correct, the data are clearly presented and discussed in the light of the latest concept of the problem. Thus, the work successfully focuses on the achieved results and they can be unambiguously compared with the data from the literature to date. Looking for a new insight into the role of probiotics in human health, a screening of the antioxidant activity of the studied lactobacillus was done. For this purpose, an original agar-diffusion method using potassium permanganate was adapted. For the active strains, the PhD student correctly notes the need for further characterization with the more discriminative DPPH and ABTS methods.

Nikola Atanasov knows the problem of preventing the development of antibiotic resistance and correctly evaluates the spectra of antibiotic sensitivity in the group of candidate probiotic strains. Thus, it performs an EFSA safety criterion assessment based on the antimicrobial resistance of newly isolated LABs.

A kind of complex nature of the evaluation of the strains was achieved with the panel of experiments proving a biotechnologically significant useful characteristic - survival during lyophilization and storage. This together, the summary of the probiotic potential of the studied strains based on the complex evaluation of the analyzes carried out for a spectrum of functional and probiotic properties was used with success in the selection of strains for inputting different formulations of a model oral health product. It shows the search for new forms and skillful combination of different tests for in situ assessment. From fundamental scientific results, he builds probiotic product models and evaluates their biotechnological relevance in line with the latest research in modern biotechnology-evidence-based health products. Impressive is not only the preservation of vitality of the selected probiotic cultures, but also the achieved safety in the given new product prototypes. I estimate the results of this final section of the work as very promising for application.

The 13 conclusions and 5 contributions made are a logical consequence of the entire development and provide objective information about the value of the conducted research. A slightly shorter, tighter statement of some of the conclusions would contribute to their accurate reflection. I accept with admiration the contributions of the development and I am convinced that they will find a real practical application. I recommend that contributions be assessed more boldly and their division into original and those of a confirmatory nature. I find contributions of a methodological nature that can be useful both in scientific-fundamental and in applied developments. The contribution of the volume of data, which can support the work of Bulgarian biotechnologists, both in research and development and in applied activities for the creation of new and better probiotic preparations, is indisputable and comprehensive.

## **ABSTRACT, CRITICAL NOTES AND QUESTIONS**

The auto-abstract, as well as the entire dissertation work are very well designed and I have no critical remarks. I would ask the following question to Nikola Atanasov, who proves with the dissertation, that he is a well-rounded and innovatively thinking biotechnologist:

*"Which product can be successfully implemented, why and how will it differ from the probiotic products on the market?"*

The merits of the work can also be judged by the presented 3 publications in journals with IF and impact rank referenced in SCOPUS, of which the doctoral student is the first author. This proves indisputably his authorship and personal contribution. I would recommend that a publication be made with the data from the bioinformatics analyzes of the whole-genome sequencing of lactobacilli isolated from the oral cavity of healthy volunteers.

## **CONCLUSION**

In conclusion, I would like to emphasize that the material is up-to-date, voluminous and fully meets the requirements of the low-ZRASRB and its Regulations. The topic is relevant, the research was carried out methodically correctly, the obtained results are a solid basis for further scientific and applied developments. The conclusions are a natural consequence of extensive

and highly scientific research. Original scientific and applied contributions stand out. It shows the excellent preparation of the doctoral student, both in the work and in its presentation.

All this gives me estates for an overall excellent assessment of the dissertation work, on the basis of which I propose to the esteemed jury to vote positively for awarding the educational and scientific degree "*Doctor*" in the field of higher education **5. Technical sciences, Professional direction - 5.11. Biotechnologies (DP. Technology of biologically active substances** DP Technology of biologically active substances of full-time doctoral student **Nikola Nikolov Atanasov.**

Sofia  
24.07.2024

Reviewer: .....  
(prof. Svetla Danova, DS.c